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Attorney Docket No. 15966-557 CIP1 (CURA-57 CIP1)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BOARD OF PATENT APPEALS AND INTERFERENCES

Art Unit:	1647
Examiner:	Christine J. Saoud
Appellants:	Jeffers *et al.*
Serial No.:	09/609,543
Filed:	July 3, 2000
For:	NOVEL FIBROBLAST GROWTH FACTOR AND NUCLEIC ACIDS ENCODING SAME

Boston, MA 02111
February 23, 2004

Board of Patent Appeals and Interferences
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BRIEF ON APPEAL

Appellants file this Appeal Brief with the Board of Patent Appeals and Interferences (*i.e.*, the "Board"), in triplicate, pursuant to 37 C.F.R. § 1.192(a), in support of their Notice of Appeal, dated April 14, 2003, and in further response to the Notice of Non-Compliance with 37 C.F.R. 1.192(c), dated January 21, 2004. This Brief is being filed on or before February 21, 2004, and is hereby timely filed. No extension of time is believed necessary. A check in the amount of $160.00 (Check No. 16667) was filed July 14, 2003, to cover the fee for filing a brief in support of an appeal required under 37 C.F.R. § 1.17(c). No further fee is believed due at this time. 37 C.F.R. §1.192(d).

The Commissioner is authorized to charge any fees that may be due, or to credit any overpayment, to Deposit Account No. 50-0311, Reference 15966-557 CIP1 (Cura-57 CIP1).

This Brief has the following Exhibits:

Exhibit 1: Jeffers, *et al*, 2002 *Gastroenterology* 123: 1151-1162;

Exhibit 2: Press Release announcing the FDA approval of CuraGen's (the assignee of this application) Investigational New Drug application to initiate human clinical trials using FGF-CX to treat oral mucositis; and

Exhibit 3: Copy of Form 1449 and postcard filed July 18, 2003, with IDS.

LIST OF CASES CITED

In re Gottlieb 328 F.2d 1016 (CCPA)

In re Brana 51 F.3d 1560 (Fed.Cir. 1995)

In re Jolles, 628 F.2d 1322 (C.C.P.A. 1980)

Brooktree Corp. v. Advanced Micro Devices, Inc., 977 F.2d 1555 (Fed. Cir. 1992)

REAL PARTY IN INTEREST

The real party in interest in this Appeal is CuraGen Corporation of New Haven, Connecticut, the sole assignee of each inventor's complete interest to all inventions described in the present application.

RELATED APPEALS AND INTERFERENCES

Appellants are unaware of any other appeal or interference proceedings that will directly affect, be directly affected by, or have a bearing on the Board's decision in the pending appeal.

STATUS OF CLAIMS

Pending claims 1, 5, 41, 46, 63 and 64, as set forth in the Appendix, are before the Board. Each of these claims has been given a Final Rejection by the Examiner.

STATUS OF AMENDMENTS

Appellants have brought this action before the Board in lieu of the position taken by the Examiner in the Examiner's Office Action of December 12, 2002 in which the claims now pending before the Board were finally rejected. The pending claims stand as amended on October 7, 2002, in response to the April 17, 2002, Examiner's non-final office action.

Prior to submitting this Brief on Appeal to the Board, and in response to the Examiner's Action of December 12, 2002, Appellants filed a Response and Amendment with the Examiner on February 6, 2003 in which they canceled claims drawn to non-elected subject matter. An Examiner's Advisory Action, mailed on March 4, 2003 ("First Advisory Action"), indicated that the Response and Amendment would be entered for purposes of Appeal, but that it did not place the application in condition for allowance because the asserted use of "promoting growth of cells in the lining of the GI tract in order to treat intestinal inflammation and ulcers" is not specifically recited in the specification as filed because it was but one in a list of unrelated uses.

Subsequently, Appellants filed a Supplemental Response requesting reconsideration, together with their Notice of Appeal, on April 14. 2003. In a Supplemental Advisory Action mailed on May 14, 2003 ("Supplemental Advisory Action"), the Examiner indicated that the April 14, 2003, Supplemental Response was entered but did not place the application in condition for allowance because: "[I]t does not support a substantial utility of the claimed invention as originally filed. Use for treatment of ulcers was not substantial at the time it was filed." An Appeal Brief was filed July 14, 2003 ("First Appeal Brief"), presenting Appellants arguments to the Board.

In a Notification of Non-Compliance with 37 C.F.R. 1.192(c) dated January 21, 2004, the Examiner indicated that (i) the First Appeal Brief did not contain a concise explanation of the claimed invention, referring to the specification by page and line number and to drawing, if any, by reference characters under 37 C.F.R. 1.192(c)(6); and (ii) the IDS filed July 18, 2003, was missing, although the references have been received. In addition, another copy of the IDS listing the references was requested. Appellants have checked the PAIR website and

determined that the Form 1449 is the misplaced document for which a copy is being requested. Therefore, a copy of the Form 1449 filed with the July 18, 2003, IDS is attached hereto as Exhibit 3.

SUMMARY OF INVENTION

The application now before the Board is a continuation-in-part of co-pending United States Patent Application Serial No. 09/494,585, filed January 31, 2000, which in turn claims priority from the filing date of United States Patent Application Serial No. 60/145,899, of July 27, 1999, now abandoned. The subject matter of the application before the Board, namely an isolated FGF-CX polypeptide of SEQ ID NO:2, has been disclosed and described since the January 31, 2000, filing date.

The claims of the present application are directed to a novel polypeptide having homology to members of the Fibroblast Growth Factor (FGF) family of proteins. *See, e.g.*, specification, p. 6 ln 22 through p. 7 ln 11, p. 8 ln 15-16; FIGS. 2-9. Specifically, these peptides are named Fibroblast Grown Factor-CX (*i.e.*, "FGF-CX"). *Id.* p. 8, ln 16-18. An example of a specific FGF-CX polypeptide, *i.e.*, the FGF-CX polypeptide that is named in the claims involved in this appeal, is the polypeptide comprising the amino acid sequence of SEQ ID NO:2 as defined within the claims and specification of the application now before the Board in this matter. *Id.* p. 6 ln 19-21, p. 90 ln 10 through p. 91 ln 7, FIG. 1. Applicants show that FGF-CX stimulates proliferation of fibroblasts *in vitro* and *in vivo* (p.102 ln.1 through p.104 ln.18; FIGS. 16-19), and disclose that the FGF-CX polypeptide has utility in promoting growth of cells in the vicinity of a wound, cells in the vascular system, cells involved in hematopoiesis, cells involved in erythropoiesis, cells in the lining of the gastrointestinal tract, and cells in hair follicles (p.5 ln.15-21).

In one embodiment, the FGF-CX polypeptide comprises conservative amino acid substitutions. *See*, p.26 ln 3 through p.27 ln 29; p. 32 ln 21 through p. 36 ln 4, including Table 2 on p. 33-34. In another embodiment, FGF-CX retains the conserved amino acids of the FGF family motif (p.91 ln.3-6, FIG. 13) and a hydrophobic transport domain (p.15 ln.11-

14; p.16 ln.13-16; FIG. 10). The FGF-CX composition may include a pharmaceutically acceptable carrier (p.64 ln.8-24) and may be provided in a kit (p.3 ln.10-12). The FGF-CX polypeptide may also comprise post-translational modifications other than proteolytic cleavage. *Id.*p.11 ln.15-21; p.94 ln 9 through p.95 ln.25.

As fully disclosed and described within the above-captioned application, the working examples on page 101, line 12, to page 104, line 18, clearly demonstrate that an FGF-CX protein as claimed is useful in stimulating cell growth, including the growth of fibroblasts, and is a therapeutic target having a promoting role in tumor progression.

ISSUES BEFORE THE BOARD

Rejection Under 35 U.S.C. § 101

Whether Claims 1, 5, 41, 46, 63 and 64 fail to meet the specific, substantial, or credible utility requirement under 35 U.S.C. § 101.

Rejections Under 35 U.S.C. § 112 ¶ 1

Whether Claims 1, 5, 41, 46, 63 and 64 fail to meet the enablement requirement under 35 U.S.C. § 112, ¶ 1, given the rejection under 35 U.S.C. §101.

GROUPING OF CLAIMS

The pending claims all recite an isolated polypeptide comprising an amino acid sequence shown in SEQ ID NO:2. To the extent that the Examiner has applied a utility rejection to this FGF-CX polypeptide of SEQ ID NO:2, these claims stand or fall together, as discussed below.

ARGUMENTS

The pending claims were entered in Appellants' October 7, 2002 response to the April 17, 2002, non-final Office Action. The only issues remaining are whether the Appellants had

met their burden of showing specific, substantial and credible utility at the time of filing the application.

The Claimed Invention

The invention described and claimed in the application before the Board relates to an isolated polypeptide comprising an amino acid sequence shown in SEQ ID NO:2. This polypeptide is alternatively referred to throughout the specification by the term "FGF-CX" or "FGF-20X". The polypeptide is also specifically referred to in each of the claims before the Board by its specific ID number.

Appellants have organized this Brief on Appeal first to provide reasons why the Examiner's rejection under 35 U.S.C. § 101 is Improper; and second to provide reasons why the Examiner's rejection under 35 U.S.C. § 112 ¶ 1 is Improper.

1. Appellants believe that the Examiner's rejection of Claims 1, 5, 41, 46, 63 and 64 under 35 U.S.C. § 101 is improper for the following reasons:

Claims 1, 5, 41, 46, 63 and 64 have been finally rejected as lacking utility "because the claimed invention is drawn to an invention with no apparent or disclosed specific and substantial credible utility" (page 3 of the December 12, 2002 Examiner's Action). In the First Advisory Action, the Examiner contends that, because multiple utilities are recited in the specification, no "specific" utility has been recited. Appellants disagree.

On pages 3 and 4 of the December 12, 2000 Final Office Action, the Examiner had set forth multiple reasons for rejecting Appellants' showing of specific examples of utility contained throughout the specification as initially filed.

The record is clear that the specification makes a specific assertion of utility for the claimed invention, i.e., the polypeptide comprising the novel FGF-CX protein of SEQ ID NO:2 to which each of the claims before the Examiner and the Board is limited.

The proteins of this invention may be used to stimulate cell growth, including especially growth of fibroblasts and epithelial cells in the linings of the gastrointestinal tract. Stimulation of cell growth is described in the specification as filed on pages 102 to 104, line

18. The Board's attention is directed, for example, to Example 10 on page 103 where it is expressly stated that the purpose of Example 10 was "To determine if recombinant FGF-CX induces cell proliferation...", and "It was found that FGF-CX induces about a 3-fold increase in cell number relative to control protein in this assay (Fig. 17)". Example 10 concludes with the statement: "These results show that FGF-CX acts as a growth factor and suggest that recombinant FGF-CX mediates the morphological transformation of NIH 3T3 cells". Appellants believe that this teaching is sufficient to provide an assertion of utility for the present invention, that the assertion identifies a specific utility which would be considered by those skilled in the art to be a substantial utility, and furthermore that the assertion of this specific and substantial utility is also credible. As such, any rejection under Sections 101 and 112, paragraph 1 of the patent code cannot be maintained. Accordingly, Appellants request the Board to find that the specification expressly provides for a specific, substantial credible utility, and to withdraw the rejection.

In addition, Appellants assert that the polypeptide comprising SEQ ID NO:2 has a credible utility in promoting growth of cells in the lining of the gastrointestinal tract in order to treat intestinal inflammation and ulcers. This demonstrated utility was discussed during a telephone conference with the Examiner on January 9, 2003. This activity is disclosed in the specification as it was originally filed (at page 4, lines 21 to 27, and page 68, lines 24 to 27, for example) and has now been published by Jeffers, *et al*, in *Gastroenterology* 123:1151-1162 (2002) which was filed with the Examiner together with the Response mailed February 6, 2003, as Appendix A. Appellants are also providing the Board with a courtesy copy of this reference as Exhibit 1 attached to this Brief on Appeal. As discussed with the Examiner during a January 9, 2003 telephonic conference, this utility of treating ulcers and cells lining the gastrointestinal tract was disclosed in the specification as filed at various other locations, including the following:

> "The invention includes a method of promoting growth of cells in a subject ... In some embodiments, the cells whose growth is to be promoted may be ... cells in the lining of the gastrointestinal tract." See page 5, lines 15 to 21.

> "FGF-CX can also be used to stimulate fibroblasts (for accelerating healing of ... ulcers)". See page 77, lines 29 and 30.
>
> "The proteins of the invention may be used to stimulate cell growth and cell proliferation in conditions in which such growth would be favorable. An example would be [in] ... linings of the gastrointestinal tract." See page 77, lines 26 to 29.

Appellants also describe this utility in the "Uses and Methods of the Invention" section covering pages 67 to 90 of the specification as initially filed. Particularly in the "Diagnostic Assays" subsection, extending from page 76 to 79 of the specification, Applicants provide details of stimulation of epithelial cells, glial cells, and cells found in the lining of the gastrointestinal tract. See, *e.g.*, page 76, line 29 to page 77, lines 9, and page 77, lines 26 to 31.

In the Advisory Action, the Examiner asserted that Appellants' response filed February 6, 2003, did not place the application in condition for allowance because the "asserted use of 'promoting growth of cells in the lining of the GI tract in order to treat intestinal inflammation and ulcers' is not specifically recited in the specification as filed because *it is but one in a list of unrelated uses*" (emphasis added). Appellants disagree and they request the Board to find in their favor. The fact that multiple utilities are recited in the specification does not mean that there is a lack of a specific, substantial and credible utility. As the MPEP makes clear, "[i]t is common and sensible for an applicant to identify several specific utilities for an invention." See MPEP § 2107.01. The case law is also clear. In re Gottlieb 328 F.2d 1016 (CCPA), is particularly relevant. In Gottlieb, multiple utilities were disclosed. The Court held that one specific utility was sufficient to meet the utility requirement (328 F.2d at 1018). That is all that is required here also. *See a*lso In re Brana 51 F.3d 1560 (Fed.Cir. 1995).

Appellants have submitted unequivocal evidence of record that confirms that the proteins claimed in the invention have precisely this activity. As stated above, Appellants previously made of record Appellants' published work demonstrating that administration of

FGF-CX protein in fact "enhances the growth of intestinal fibroblasts." *See* Jeffers *et al.* (citing Abstract), (Exhibit 1).

In addition, Appellants submitted a Press Release announcing the FDA approval of CuraGen's (the assignee of this application) Investigational New Drug application to initiate human clinical trials using FGF-CX to treat oral mucositis – oral mucositis is a side effect of chemotherapy and radiotherapy resulting in the degradation of mucosal tissue that can range from redness and irritation to severe ulcerations of the mouth and throat (a courtesy copy is attached as Exhibit 2). In this trial, FGF-CX is being tested for its ability to stimulate cell proliferation (specifically proliferation of fibroblasts and epithelial cells) and to counteract toxic side effects of chemotherapeutic and radiotherapeutic agents in the throat and mouth (*i.e.*, linings of the gastrointestinal tract), precisely as recited in the specification. Appellants believe that the Board must agree that this is more than is required to prove an overabundance of utility in front of the United States Patent and Trademark Office.

For the record, Appellants note that utility is also supported by the structural similarity of FGF-CX with other known members of the FGF family and specifically contains a conserved family domain and hydrophobic transport domain (see page 91, lines 3 to 7 of the specification as filed). In addition, the claimed FGF-CX polypeptide has a biological activity similar to a structurally related fibroblast growth factor-9 (FGF-9) compound already known and tested in the art for activation/proliferation of glial cells and fibroblasts (see pages 76 and 77 of the specification as filed along with FIGS. 4 and 5). Other known FGFs have been shown to be useful in the stimulation of wound healing (see, for example, United States Patent 5,804,213). In addition, case law holds as valid a utility for claimed compounds based on structural features similar to the facts in the instant application, for example, the Court finding utility for claimed compounds having close structural relationship to other compounds known to be useful in cancer therapy in In re Jolles, 628 F.2d 1322 (CCPA 1980); or stating that although it may be true that minor changes in chemical compounds can radically alter their effects, evidence of success in structurally similar compounds is relevant in determining whether one skilled in the art would believe an asserted utility in In re Brana 51 F.3d 1560

(Fed. Cir. 1995). Thus, the particular utility in the present matter before the Board, namely diagnosing and treating cell proliferation associated disorders such as wound healing associated with oral mucositis for FGF-CX, is fully supported and consistent with generally accepted scientific principles as well as in accordance with current case law.

As shown above, the specification clearly discloses utility of the proteins of this invention for treating ulcers and cells lining the gastrointestinal tract in accordance with the requirement of the patent statutes. As illustrated in the attached Jeffers article (Exhibit 1), the proteins of this invention have a demonstrated therapeutic activity of treating intestinal inflammation in both animal *in vivo* studies and *in vitro* studies using human cell lines. In the scientifically acceptable murine-colitis model, it was shown that prophylactic administration of FGF-CX (corresponding to SEQ ID NO:2 of the claims before the Board) significantly reduced the severity and extent of mucosal damage; in the scientifically accepted rat small bowel ulceration/inflammation model, administration of FGF-CX was shown to reduce small intestinal weight gain, necrosis, inflammation, and weight loss; and in *in vitro* studies it was demonstrated that FGF-CX stimulated cell growth and restitution in human intestinal fibroblast cell lines. Accordingly, FGF-CX (SEQ ID NO:2) has been shown to have a specific, substantial, and credible utility of treating intestinal disorders. This utility was specifically disclosed in the specification as originally filed. Appellants therefore request that the Board withdraw the rejection.

2. Appellants believe that the Examiner's rejection of Claims 1, 5, 41, 46, 63 and 64 under 35 U.S.C. § 112 ¶ 1 (Lack of Enablement) is improper for the following reasons:

Claims 1, 5, 41, 46, 63 and 64 were finally rejected under 35 U.S.C. § 112 ¶ 1 for lack of enablement. This rejection is attendant to the rejection based on the above § 101 lack of utility. Since the Examiner alleges that the claimed invention lacks utility, it follows that one skilled in the art would not know how to use the claimed invention. Appellants do not agree with this determination, and they respectfully request the Board to find in their favor.

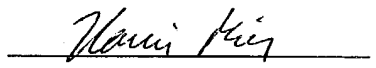
The Examiner has implicated a "how to use" utility-based § 112 ¶ 1, rejection. This cannot stand. In order for the Examiner's position to be upheld on the utility-based § 112, ¶ 1, non-enablement rejection, the specific factual showing must represent one of those rare instances meeting the stringent criterion of being "totally incapable of achieving a useful result" Brooktree Corp. v. Advanced Micro Devices, Inc., 977 F.2d 1555 (Fed. Cir. 1992), as discussed in the Legal Analysis accompanying the Utility Guidelines (M.P.E.P. § 2107). The only instances in which the Federal courts have found a lack of patentable utility were where, "based upon the factual record of the case, it was clear that the invention *could and did not work* as the inventor claimed it did." M.P.E.P. § 2107 (emphasis added). These rare cases have been ones in which the applicant either (a) failed to disclose any utility for the invention, or (b) asserted a utility that could be true only "if it violated a scientific principle, such as the second law of thermodynamics, or a law of nature, or was wholly inconsistent with contemporary knowledge in the art." M.P.E.P. § 2107.01. That is simply not the case here -- as is evidenced from the Jeffers paper, and the FDA's approval of the IND (Exhibits 1 and 2, respectively).

Appellants have overcome the Office's rejection under § 101 to the claims as amended above. Accordingly, the rejection under § 112, ¶ 1 must also fall. Withdrawal of the rejection is respectfully requested.

CONCLUSION

In view of the foregoing comments and reasons, Appellants request that the Board of Patent Appeals and Interferences find in their favor and overturn the Examiner's Final Rejection and return their application to the Examiner with an indication that all claims before the Board in this matter fully comply with the requirements of 35 U.S.C. § 101 and 35 U.S.C. § 112 first paragraph, and are thus in condition for allowance.

Respectfully submitted,



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APPENDIX: CLAIMS ON APPEAL

1. (Previously amended) An isolated polypeptide comprising an amino acid sequence shown in SEQ ID NO:2.

5. (Previously amended) The polypeptide of claim 1, said polypeptide further comprising at least one conservative amino acid substitution, wherein said polypeptide is a full length polypeptide that retains functional growth factor-like properties of SEQ ID NO: 2, retains the conserved amino acids of the FGF family motif located at residues 125, 127, 129, 136, 137, 139, 141 and 148, and retains the hydrophobic transport domain between residues 92-120, wherein the residues are numbered with respect to SEQ ID NO:2.

41. (Previously amended) A composition comprising the polypeptide of claim 1 and a pharmaceutically acceptable carrier.

46. (Previously amended) A kit comprising in one or more containers a composition comprising the polypeptide of claim 1 and a pharmaceutically acceptable carrier.

63. (Previously added) The polypeptide of claim 1, the polypeptide further comprising a post-translational modification other than a proteolytic cleavage.

64. (Previously added) The polypeptide of claim 63, wherein the post-translational modification is at least one modification chosen from the group consisting of phosphorylation and N-myristoylation.

GASTROENTEROLOGY 2002;123:1151–1162

A Novel Human Fibroblast Growth Factor Treats Experimental Intestinal Inflammation

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Background & Aims: We recently identified a novel member of the human fibroblast growth factor (FGF) family of signaling molecules, designated FGF-20. In the present study, we examined the activity of this protein in 2 animal models of acute intestinal inflammation and in mechanistic studies in vitro. *Methods:* In vivo experiments consisted of a murine dextran sulfate sodium (DSS) model of colitis and a rat indomethacin model of small intestinal ulceration/inflammation. Cell growth, restitution, gene expression (cyclooxygenase-2 [COX-2] and intestinal trefoil factor [ITF]), and prostaglandin E_2 (PGE_2) levels were examined in vitro. *Results:* In the DSS-colitis model, prophylactic administration of FGF-20 significantly reduced the severity and extent of mucosal damage as indicated by a 55%–93% reduction in luminal blood loss, distal colonic edema, histologic inflammation, and epithelial cell loss relative to animals administered vehicle control. No toxicity was noted during administration of FGF-20 to normal controls. In addition, therapeutic administration of FGF-20 enhanced survival in this model. In the indomethacin–small bowel ulceration/inflammation model, administration of FGF-20 reduced small intestinal weight gain, necrosis, inflammation, and weight loss (36%–53% relative to vehicle control). In vitro studies demonstrated that FGF-20 stimulates growth, restitution, mRNA expression of COX-2 and ITF, and PGE_2 levels in human intestinal epithelial cells and enhances the growth of human intestinal fibroblasts. *Conclusions:* FGF-20, having demonstrated therapeutic activity in 2 experimental models of intestinal inflammation, represents a promising new candidate for the treatment of human inflammatory bowel disease.

Inflammatory bowel disease (IBD) comprises a spectrum of immune-mediated chronic gastrointestinal disorders, including ulcerative colitis and Crohn's disease.[1–3] In IBD, the integrity of the intestinal mucosa is compromised, and healing of the surface epithelium is accomplished via epithelial cell migration ("restitution"), proliferation, and differentiation.[4] Stimulated fibroblasts are believed to play a role in the healing process.[4]

Although many treatments for IBD exist, additional therapeutic approaches are needed because many patients either do not respond to current options or develop significant side effects to medications, thereby precluding their continued use. Because of the inadequacy of current therapies, some IBD patients with refractory disease undergo surgery to remove a portion of the intestine. A new agent that has fewer side effects than current approaches, has sustainable efficacy in patients unresponsive to available drugs, targets therapeutic mechanisms distinct from current medications, and eliminates the need for surgery would offer clinical and pharmacoeconomic benefits. Because the integrity of the intestinal mucosa is breached in IBD, thereby potentiating the uptake of injurious luminal bacterial antigens and cell wall polymers,[1] it follows that agents that facilitate or accelerate epithelial repair may be therapeutically useful. No currently marketed products fall into this category.

One relevant class of agents for the repair of intestinal epithelium is that of the peptide growth factors, including, among others, members of the fibroblast growth factor (FGF) family.[5,6] FGF receptors are present on intestinal epithelium,[7,8] and enhanced expression of various FGF family members has been demonstrated in the intestines of IBD patients,[9–12] suggesting a potential endogenous reparative role for this family of growth factors after injury caused by inflammatory processes.

Abbreviations used in this paper: COX-2, cyclooxygenase-2; DMEM, Dulbecco's modified Eagle medium; DSS, dextran sulfate sodium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FGF, fibroblast growth factor; IBD, inflammatory bowel disease; ITF, intestinal trefoil factor; PBS, phosphate-buffered saline; PGE_2, prostaglandin E_2; RT-PCR, reverse-transcription polymerase chain reaction.

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Support for this theory comes from in vitro and in vivo studies demonstrating that FGFs enhance the restitution and proliferation of intestinal epithelium.[7,13–15] Assuming that naturally occurring reparative factors such as FGFs are present in suboptimal quantities in the intestines of IBD patients, it follows that supplementation with these factors may promote healing and thus alleviate the symptoms associated with IBD. FGFs have in fact shown promise in animal models of IBD.[13,16–18]

We recently identified and characterized a novel member of the human FGF family that we designate FGF-20.[19] This factor interacts with multiple FGF receptors and displays mitogenic activity on fibroblasts and epithelial cells. In the present study, we examined the in vivo effects of FGF-20 in 2 rodent models of IBD: dextran sulfate sodium (DSS) treatment of mice to induce an ulcerative colitis-like syndrome and indomethacin treatment of rats to induce ulceration and inflammation of the small bowel, as is seen in Crohn's disease. We also performed in vitro studies to explore the mechanisms of action of FGF-20.

Materials and Methods

Purification of FGF-20

The human FGF-20 cDNA[19] was cloned into pETMY, a modified pRSET vector (Invitrogen, San Diego, CA). The resulting construct encodes amino acids 2–211 of FGF-20 preceded by the sequence MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKDRWGS, which contains a histidine tag used for purification purposes, as well as additional vector-encoded residues. The vector was transformed into *Escherichia coli* strain BL21 (Novagen, Madison, WI), which was grown to an optical density of 0.6 and infected with CE6 bacteriophage lambda (Novagen) at a multiplicity of infection of 5. The infected bacterial culture was further incubated for 3 hours at 27°C, obtained by centrifugation (4000×*g* for 15 minutes at 4°C), resuspended in phosphate-buffered saline (PBS) + 0.5 mol/L NaCl + 1.0 mol/L L-arginine, and disrupted with 2 passes through a microfluidizer at 8000 pounds per square inch. Cell debris was removed by centrifugation (10,000 × *g* for 25 minutes at 4°C) and discarded. The resulting supernatant containing the FGF-20 protein was clarified by filtration through a 0.22-μm low protein-binding filter and loaded onto a column containing nickel-charged Sepharose (Pharmacia Biotech, Piscataway, NJ). The column was washed with PBS + 0.5 mol/L NaCl + 1.0 mol/L L-arginine, and bound protein was eluted with a linear gradient of 0–0.5 mol/L imidazole. Fractions containing FGF-20 were pooled, dialyzed at 4°C against PBS + 1.0 mol/L L-arginine, and loaded onto a column containing uncharged Sepharose (Pharmacia Biotech). FGF-20 was captured in the flowthrough, dialyzed at 4°C against PBS + 1.0 mol/L L-arginine, and sterilized by passage through a 0.22-μm low protein-binding filter. Purified FGF-20 had an endotoxin level of ≤25 endotoxin units/mg as determined by the Limulus amebocyte lysate assay (BioWhittaker, Walkersville, MD).

Bromo-Deoxy-Uridine Assay

NIH 3T3 murine embryonic fibroblasts and Balb/MK murine keratinocytes were cultured in 96-well plates to approximately 100% confluence in growth media (NIH 3T3 cells: Dulbecco's modified Eagle medium [DMEM] + 10% bovine calf serum [Invitrogen]; Balb/MK cells: keratinocyte-SFM [Invitrogen]). Before adding FGF-20, Balb/MK cells were prestarved for 24 hours in basal keratinocyte media. FGF-20 was added to cells for 18 hours in the appropriate basal media supplemented with 0.1% bovine serum albumin, and the bromo-deoxy-uridine assay was performed according to the manufacturer's specifications (Roche Molecular Biochemicals, Indianapolis, IN) using a 3 hour bromo-deoxy-uridine incorporation time.

Animals

Mice. Six- to 8-week-old female Balb/c mice weighing 20–22 g were obtained from Harlan Labs (Indianapolis, IN) for use in the DSS model. Between 3 and 5 animals were housed per cage in polycarbonate cages with filter tops and given mouse chow (Harlan Teklab, Madison, WI) and tap water ad libitum. Animals were acclimated for 6 days before experimental use and were sacrificed by CO_2 inhalation at the end of the study.

Rats. Female Lewis rats weighing 175–200 g were obtained from Harlan Labs for use in the indomethacin model. Four animals were housed per cage and given Harlan Teklab rat chow and tap water ad libitum. Animals were acclimated for 8 days before experimental use. At the end of the study, animals were anesthetized with isoflurane and sacrificed by cervical dislocation after blood collection.

Murine DSS Model

DSS (Spectrum Chemicals, Gardena, CA) working solutions were freshly made every other day in tap water and stored at 4°C. FGF-20 was diluted in PBS + 1.0 mol/L L-arginine, and the vehicle solution consisted of PBS + 1.0 mol/L L-arginine. Intraperitoneal (IP) and subcutaneous (SC) injections were both performed in volumes of 10 mL/kg using FGF-20 stocks of the appropriate concentrations so as to achieve the desired final concentration of FGF-20 (5, 1, or 0.2 mg/kg, as indicated in the Results section and in the Figures). At necropsy, the colon was removed, and colon blood content was scored according to the following criteria: 0, normal to semisolid stool, no blood; 1, normal to semisolid stool, blood-tinged; 2, semisolid to fluid stool with definite evidence of blood; 3, bloody fluid. For histopathologic examination, 3 distal colonic regions spaced approximately 1 cm apart were collected into 10% neutral buffered formalin, processed for paraffin embedding, sectioned, and stained with hematoxylin and eosin. Each section was scored for various parameters, and the mean of the scores for each of the regions was determined.

Submucosal edema was quantitated by measuring the distance from the muscularis mucosa to the internal border of the outer muscle layer. Inflammation (foamy macrophage, lymphocyte, and polymorphonuclear, cell infiltrate) was assigned a severity score according to the following criteria: 0, normal; 1, minimal; 2, mild; 3, moderate; 4, marked; 5, severe. Glandular epithelial loss and surface epithelial loss were scored using the following criteria: 0, normal; 1, 1%–10% of the mucosa affected; 2, 11%–25% of the mucosa affected; 3, 26%–50% of the mucosa affected; 4, 51%–75% of the mucosa affected; 5, 76%–100% of the mucosa affected. The 3 important scored parameters (inflammation, glandular epithelial loss, and surface epithelial loss) were combined to arrive at an overall histopathology score that indicates the overall damage and could have a maximum score of 15. For each animal, 3 distal colonic regions spaced approximately 1 cm apart were scored, and the means of the scores for each of the regions were determined.

Rat Indomethacin Model

To induce disease, indomethacin (Sigma, St. Louis, MO) was prepared in 5% sodium bicarbonate to 7.5 mg/mL and injected SC into rats on 2 consecutive days in a volume of 1 mL/kg so as to achieve the desired final concentration of 7.5 mg/kg/dose.[13] FGF-20 was diluted in PBS + 1.0 mol/L L-arginine, and the vehicle solution consisted of PBS + 1.0 mol/L L-arginine + 5 mg/mL bovine serum albumin. Intravenous (IV) tail vein injections were performed in a volume of 1 mL/kg using FGF-20 stocks of the appropriate concentrations so as to achieve the desired final concentration of FGF-20 (5, 1, or 0.2 mg/kg as indicated in the Results section and in the Figures). At necropsy, a 10-cm section of the distal jejunum in the area at risk for lesions was removed and weighed. This jejunum fragment was then used to obtain 5 approximately equally spaced sections that were collected into 10% neutral buffered formalin, processed for paraffin embedding, sectioned, and stained with hematoxylin and eosin for histopathologic examination. Necrosis was scored according to the following criteria: 0, normal; 1, 1%–10% mucosal necrosis; 2, 11%–25% mucosal necrosis; 3, 26%–50% mucosal necrosis; 4, 51%–75% mucosal necrosis; 5, 76%–100% mucosal necrosis. Inflammation was scored according to the following criteria: 0, none; 1, minimal inflammation in mesentery and muscle or lesion; 2, mild inflammation in mesentery and muscle or lesion; 3, moderate inflammation in mesentery and muscle or lesion; 4, marked inflammation in the lesion; 5, severe inflammation in the lesion.

Growth Assay

CCD-18Co normal human colonic fibroblasts and FHs 74 Int normal human small intestinal epithelial cells were plated in 6-well plates to approximately 25% confluence in growth media and allowed to attach overnight (CCD-18Co cells: DMEM + 10% FBS [Invitrogen]; FHs 74 Int cells: DMEM + 10% FBS + nonessential amino acids [Invitrogen] + sodium pyruvate [Invitrogen] + 1 mmol oxalacetic acid [Sigma] + 0.2 U/mL insulin [Invitrogen]). The next day, the growth media was removed and replaced with a 1:1 mixture of DMEM (without or with FGF-20)/growth media. The cells were fed with fresh factor after 3 days and counted after 6 days.

Wounded Monolayer Repair Assay

An in vitro healing assay was performed using a modified version of a published method.[13,20] Briefly, reference lines were drawn horizontally across the outer bottom of 24-well plates. HT-29 and Caco-2 human colon carcinoma cells were plated and grown to confluence in DMEM + 5% FBS, and then incubated for 24 hours in DMEM + 0.1% FBS. Linear "wounds" were made with a sterile plastic pipette tip perpendicular to the lines on the bottom of the wells. Then 10% FBS (positive control) or FGF-20 was added, and the size of the wound was measured microscopically at various times at predetermined locations corresponding to the reference lines.

Determination of Cyclooxygenase-2 and Intestinal Trefoil Factor Gene Expression by Reverse-Transcription Polymerase Chain Reaction

Cells (HT-29 and Caco-2) were plated and grown to confluence in DMEM + 5% FBS, and then incubated for 24 hours in DMEM + 0.1% FBS. FGF-20 (100 ng/mL) was then added, and total RNA was obtained from the cells after various times using Trizol (Invitrogen) according to the manufacturer's instructions. RNA was reverse-transcribed using 2 μg of total RNA, 15 U of RNA inhibitor, first-strand synthesis buffer (Invitrogen), 5 mmol deoxynucleoside triphosphate (Pharmacia, Upsala, Sweden), 125 pmol random hexamer primers (Pharmacia), and 125 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen) in a final volume of 25 μL. The reaction was performed for 1 hour at 39°C, followed by 7 minutes at 93°C and 1 minute at 24°C, and then slowly cooled to 4°C for 20 minutes. Reverse transcription-polymerase chain reaction (RT-PCR) was performed in a volume of 50 μL containing 5 μL of reverse transcriptase mixture, 1× Taq buffer, 5 pmol of each primer, 2.5 mmol deoxynucleoside triphosphate, and 1 unit of Taq polymerase. The primers used to amplify human cyclooxygenase-2 (COX-2), intestinal trefoil factor (ITF), and β-actin were as follows:

COX-2: sense, 5′-AGATCATCTCTGCCTGAGTATCTT-3′; antisense, 5′-TTCAAATGAGATTGTGGGAAAATT-GCT-3′

ITF: sense, 5′-GTGCCAGCCAAGGACAG-3′; antisense, 5′-CGTTAAGACATCAGCCTCCAG-3′

β-actin: sense, 5′-CCAACCGCAAGAAGATGA-3′; antisense, 5′-GATCTTCATGAGGTAGTCAGT-3′.

RT-PCR was carried out in a Perkin-Elmer 9600 cycler (Perkin-Elmer, Wellesley, MA) programmed for 20–40 cycles to assess the linearity of the amplification. The PCR products were separated on 2% Tris-acetate/EDTA agarose gels containing gel star fluorescent dye (FMC, Philadelphia, PA). A negative from the gels was taken with an AlphaImager 2000 (Alpha Innotech, San Leandro, CA).

Determination of Prostaglandin E_2 Levels by Enzyme-Linked Immunosorbent Assay

Cells (HT-29 and Caco-2) were plated and grown to confluence in DMEM + 5% FBS, and then incubated for 24 hours in DMEM + 0.1% FBS. FGF-20 was then added, and 24 hours later the culture medium was harvested and assessed for prostaglandin E_2 (PGE_2) levels via enzyme-linked immunosorbent assay (ELISA; Assay Designs, Ann Arbor, MI).

Results

Expression, Purification, and In Vitro Activity of Recombinant FGF-20

Purified human FGF-20 was isolated from *Escherichia coli* engineered to express full-length FGF-20 protein (Figure 1*A*). The recombinant protein, which contains vector-encoded sequences and a histidine tag at the N-terminus, had a molecular weight of approximately 29 kilodaltons (Figure 1*A*, Lane 2), close to its predicted molecular weight of 27,739 daltons. Purified FGF-20 was biologically active, as demonstrated by its ability to induce DNA synthesis in murine fibroblasts (NIH 3T3) and epithelial cells (Balb/MK) at half maximal concentrations of approximately 5 ng/mL (Figure 1*B*). Similar biological activity was obtained with purified recombinant full-length FGF-20 devoid of vector-encoded sequences (data not shown).

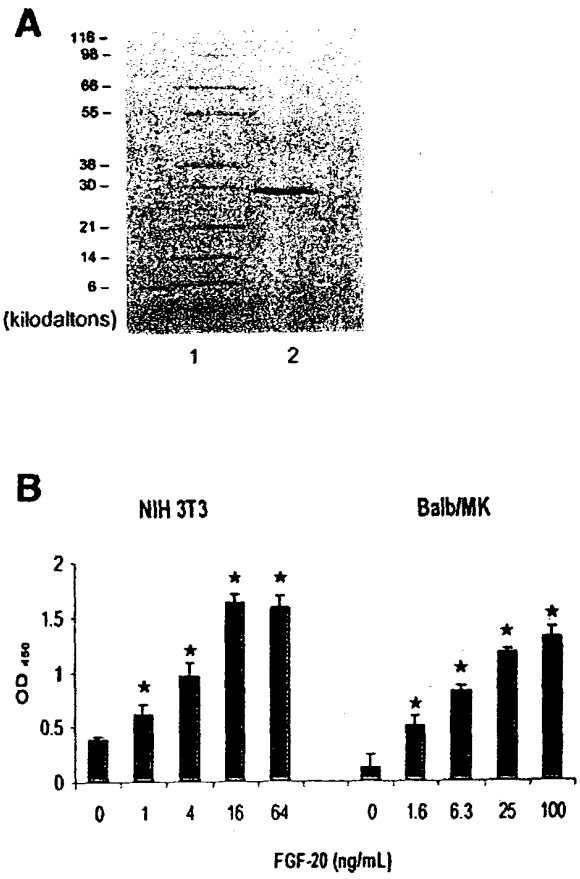

Figure 1. Purification and in vitro biological activity of FGF-20. (*A*) Full-length FGF-20 protein possessing an N-terminal histidine tag was expressed in *Escherichia coli* and purified to near homogeneity by nickel chromatography. Five micrograms of FGF-20 was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (4%–20% gel) under reducing conditions and visualized with Coomassie stain (*lane 2*). *Lane 1* depicts protein standards (in kilodaltons). (*B*) NIH 3T3 murine fibroblasts and Balb/MK murine keratinocytes were incubated with purified FGF-20 at the indicated concentrations for 18 hours and analyzed by a bromo-deoxy-uridine incorporation assay. Data points represent the mean of triplicate wells ± standard deviation. Changes that are statistically different from control cells receiving no factor ($P < 0.05$ by the 2-tailed Student *t* test) are indicated with a star.

Prophylactic Administration of FGF-20 Is Active in a DSS-Mediated Murine Model of Colitis

The effect of FGF-20 on colitis was initially examined in a murine DSS-mediated disease model.[21,22] In this model, Balb/c mice exposed to DSS for 7 days developed distal colonic inflammation and edema in association with crypt and colonic glandular epithelial loss, erosion, and ulceration, leading to hemorrhage. In this study, DSS-associated effects on the proximal colon were much less severe than on the distal colon and thus are not reported. FGF-20 (5 mg/kg) administered daily via IP injections on each of the 7 days of DSS exposure significantly reduced the extent and severity of mucosal damage (Figure 2). Specifically, FGF-20 resulted in the following protective effects on the distal colon: 93% reduction of blood content scores, reflecting hemorrhagic diarrhea; 76% reduction in submucosal edema; 55% reduction in mucosal inflammation; 57% reduction in glandular epithelial loss; and 84% reduction in surface epithelial loss. FGF-20 administration also inhibited the DSS-induced decrease in colon length. Histopathology sum scores that take into consideration the parameters of inflammation, glandular epithelial loss, and erosion indicate that FGF-20 caused a 66% reduction in DSS-mediated effects on the distal colon. Finally, FGF-20 administration reduced the amount of DSS-induced weight loss by 30%. A representative histopathologic example of the protective effect of FGF-20 on the distal colon is depicted in Figure 3, which shows that FGF-20 inhibited the mucosal changes and submucosal edema associated with DSS treatment.

In a follow-up murine DSS-colitis study, we sought to verify the initial results and to determine optimal dosing in the prophylactic protocol using a SC delivery method. Mice were exposed to DSS for 7 days, and FGF-20 (5, 1,

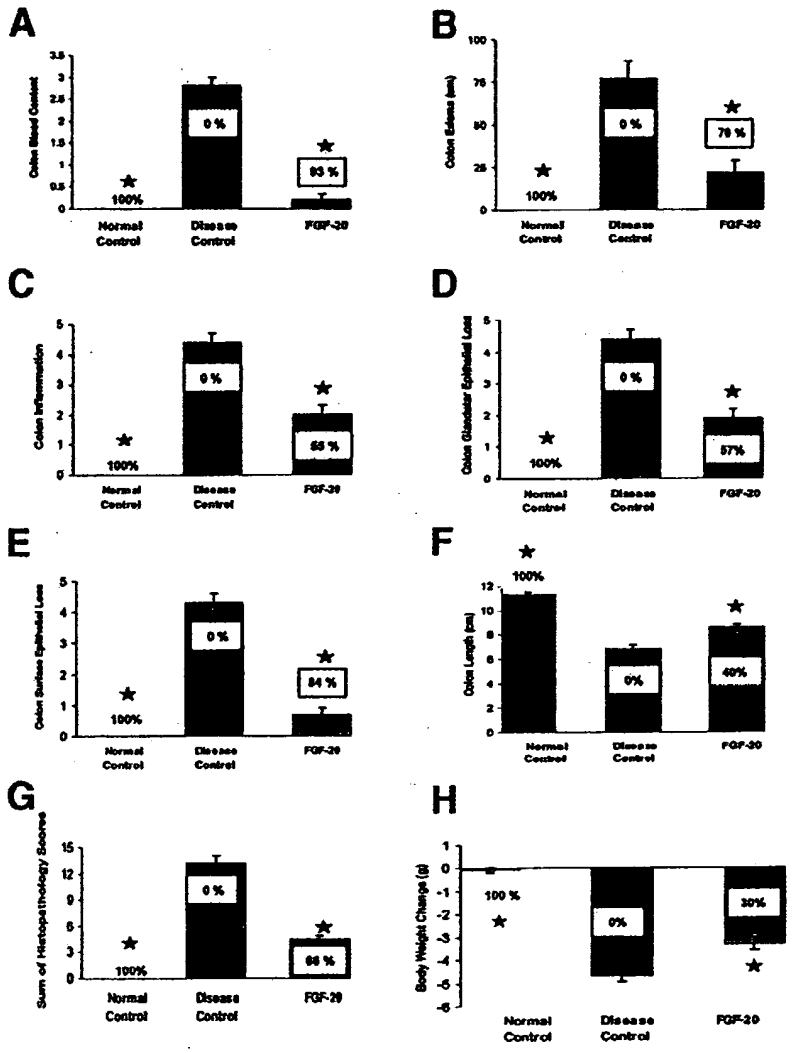

Figure 2. Effects of FGF-20 on DSS-induced colitis. To induce colitis, female Balb/c mice were exposed to 5% DSS in drinking water for 7 days (day 0 to 6). Disease control animals (n = 10) received daily IP injections of vehicle solution on each day of DSS exposure. The FGF-20 group of animals (n = 10) received daily IP injections of FGF-20 (5 mg/kg) on each day of DSS exposure. Normal control animals (n = 5) were not exposed to DSS, but did receive daily IP injections of vehicle solution on day 0 to 6. Animals were sacrificed on day 7, and the distal colon was scored for the following parameters (see Materials and Methods for details): (*A*) blood content, (*B*) submucosal edema, (*C*) mucosal inflammation, (*D*) glandular epithelial loss, (*E*) surface epithelial loss (erosion), and (*F*) length. (*G*) An overall histopathology score that takes into consideration inflammation, glandular epithelial loss, and erosion in the distal colon. (*H*) The change in total body weight from day 0 to 7. Results are reported as mean ± standard error, and the percentage changes indicated are relative to the disease control group. Changes that are statistically different from the disease control group ($P < 0.05$ by the 2-tailed Student t test) are indicated with a star.

and 0.2 mg/kg) was administered daily via SC injections on each of the 7 days of DSS exposure (Figure 4). As was seen in the initial study, FGF-20 treatment reduced the extent and severity of mucosal damage as measured by fecal blood, histologic injury, and colon length, and did so in a dose-dependent fashion with maximum protection offered at the highest FGF-20 concentration examined (5 mg/kg). No significant protective effect of FGF-20 on DSS-induced weight loss was observed in this follow-up study. Significant protection from mucosal damage was also seen with FGF-20 at 1 mg/kg, whereas 0.2 mg/kg of this factor provided little protection from DSS-induced colitis.

Similar protective effects were obtained with purified recombinant full-length FGF-20 devoid of vector-encoded sequences. Moreover, following the administration of this FGF-20 protein (5 mg/kg SC once daily for 7 days) to normal nondisease control animals, an analysis of animal weight, blood hematology/clinical chemistry, and histopathology on 28 different tissues

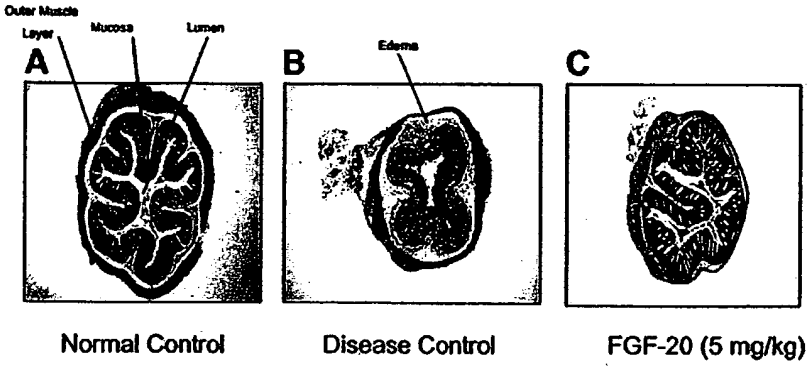

Figure 3. Effects of FGF-20 on DSS-induced colitis: histopathology. Representative sections of distal colon were collected at necropsy, preserved in formalin, stained with H&E, magnified 50×, and photographed. The groups are as described in the legend to Figure 2. Note that FGF-20 (5 mg/kg) inhibits the mucosal changes and submucosal edema associated with DSS treatment.

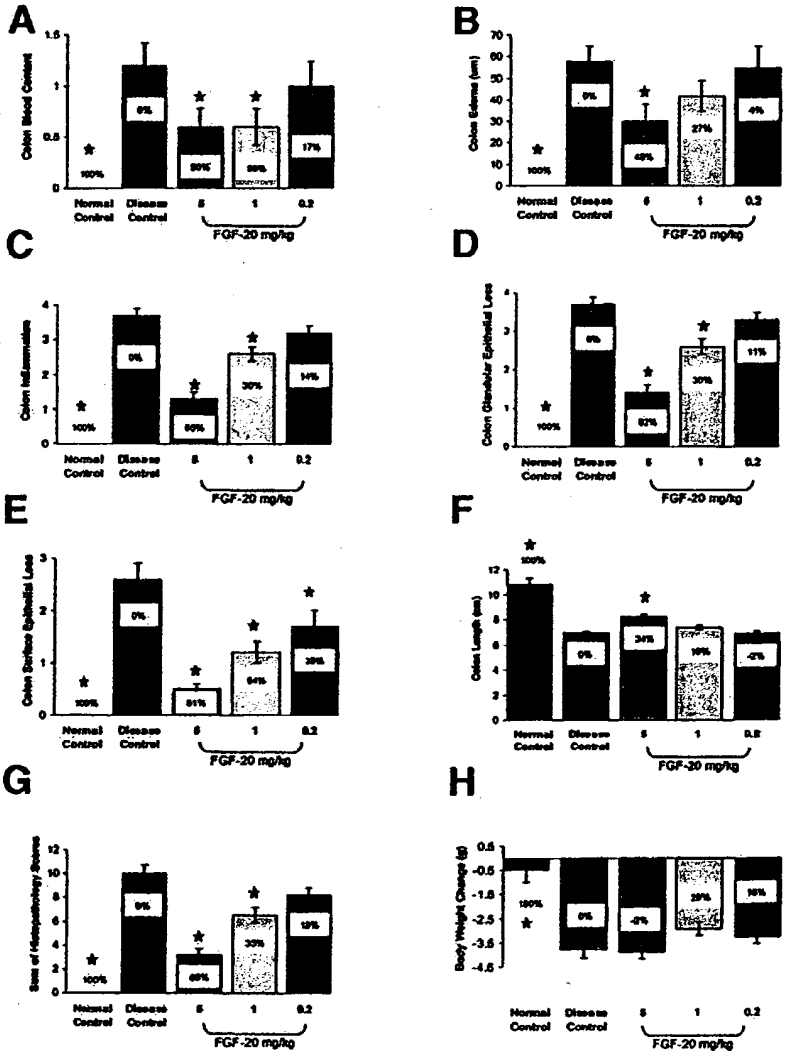

Figure 4. Effects of FGF-20 on DSS-induced colitis: dose response. To induce colitis, female Balb/c mice were exposed to 4% DSS in drinking water for 7 days (day 0 to day 6). Disease control animals ($n = 9$) received daily SC injections of vehicle solution on each day of DSS exposure. The FGF-20 group of animals ($n = 9$) received daily SC injections of FGF-20 at the indicated concentrations on each day of DSS exposure. Normal control animals ($n = 4$) were not exposed to DSS, but did receive daily SC injections of vehicle solution on day 0 to day 6. Animals were sacrificed on day 7, and the distal colon was scored for the following parameters (see Materials and Methods for details): (*A*) blood content, (*B*) submucosal edema, (*C*) mucosal inflammation, (*D*) glandular epithelial loss, (*E*) surface epithelial loss (erosion), and (*F*) length. (*G*) An overall histopathology score that takes into consideration inflammation, glandular epithelial loss, and erosion in the distal colon. (*H*) The change in total body weight from day 0 to 7. Results are reported as mean ± standard error, and the percentage changes indicated are relative to the disease control group. Changes that are statistically different from the disease control group ($P < 0.05$ by the 2-tailed Student *t* test) are indicated with a star.

revealed that the only gross tissue alteration induced by FGF-20 was increased injection site inflammation/fibroplasia. This analysis also indicated that FGF-20 induced a moderate increase in absolute neutrophils and cholesterol. (Mean absolute neutrophil counts in FGF-20-treated, vehicle control and nontreated control animals were 1405, 968, and 1130, respectively; t test P value = 0.030 for comparison of FGF-20-treated to vehicle control. Mean cholesterol counts in FGF-20-treated, vehicle control and nontreated control animals were 100, 79.2, and 77.2, respectively; t test P value = 0.024 for comparison of FGF-20-treated to vehicle control). The biological relevance of these findings remains to be determined.

Therapeutic Administration of FGF-20 Enhances Survival in the Murine DSS Model

In the experiments described previously, DSS exposure and FGF-20 administration were initiated simultaneously on day 0. In another experiment, the effect of FGF-20 administered after the initiation of DSS treatment was examined. To this end, Balb/c mice exposed to DSS for 7 days (day 0 to day 6) were injected daily SC with various concentrations of FGF-20 (5, 1, and 0.2 mg/kg) beginning on the fifth day of DSS exposure (i.e., day 4) and ending 3 days after the termination of DSS exposure (i.e., day 9). Animal survival was recorded on a daily basis, and the experiment was concluded on day 10. As shown in Figure 5, therapeutic administration of FGF-20 at 5 mg/kg enhanced survival relative to the disease control group. Thus, whereas only 44% (4 of 9) of the animals in the disease control group survived until the end of the study, 89% (8 of 9) of the animals treated with FGF-20 at 5 mg/kg survived. FGF-20 administered therapeutically at lower doses (1 and 0.2 mg/kg) had little or no effect on survival.

FGF-20 Is Active in an Indomethacin-Mediated Rat Model of Small Bowel Ulceration/Inflammation

Treatment of susceptible Lewis rats with indomethacin results in chronic small intestinal linear ulcerations bearing some similarity to those observed in Crohn's disease. This model was used to examine the ability of FGF-20 to treat discrete mucosal ulcers. To this end, Lewis rats treated with indomethacin (7.5 mg/kg SQ) for 2 days (day 0 to day 1) were injected daily IV with various concentrations of FGF-20 (5, 1, 0.2 mg/kg) beginning on the day before the initiation of indomethacin treatment (i.e., day −1) and ending 3 days after the termination of indomethacin treatment (i.e., day 4). Animals were sacrificed and examined on day 5. Administration of FGF-20 at 0.2 mg/kg resulted in the following protective effects relative to vehicle-treated disease control animals: 52% reduction in indomethacin-induced small intestine weight increase (as measured from a 10-cm section of distal jejunum taken from the area at risk), 53% reduction in histopathologic intestinal necrosis, and a 38% reduction in histopathologic intestinal inflammation (Figure 6). This concentration of FGF-20 also significantly reduced the indomethacin-induced increase in blood neutrophils by 39% and inhibited weight loss by 36%. Higher concentrations of FGF-20 (i.e., 1 and 5 mg/kg) were less active in this model than the 0.2 mg/kg dose. A representative histopathologic example of the protective effect of FGF-20 on the small intestine is depicted in Figure 7, which shows that FGF-20 inhibits the mucosal ulceration and necrosis normally associated with indomethacin treatment.

FGF-20 Enhances the Growth and Restitution of Intestinal Cells In Vitro

Because the healing of the surface mucosa involves epithelial restitution, as well as epithelial and fibroblast growth and/or activation, we examined the effect of FGF-20 on these processes in vitro. Our findings demonstrate that FGF-20 significantly enhances the growth of normal human colonic fibroblasts (CCD-18Co) and normal human intestinal epithelial cells (FHs Int 74) (Figure 8). This effect of FGF-20 was dose-dependent and resulted in a 2- to 3-fold increase in cell number over the course of 6 days of culture.

To explore the effects of FGF-20 on restitution, a wounded monolayer repair assay was performed on 2 human colonic epithelial cancer cell lines, HT-29 and Caco-2. The results of this assay demonstrate that FGF-20 significantly stimulates wound closure in a concentration-dependent manner (Figure 9). The highest FGF-20 dose examined (100 ng/mL) stimulated closure to a similar degree as that of the positive control (10% FBS). Consistent with these results, FGF-20 was also found to enhance the migration of FHs Int 74 normal human intestinal epithelial cells in a dose-dependent fashion when examined in a modified Boyden chamber assay (data not shown).

FGF-20 Stimulates COX-2 and ITF Expression and PGE_2 Levels in Colonic Cells In Vitro

We next examined the effect of FGF-20 on the mRNA expression of COX-2 and ITF, 2 genes whose

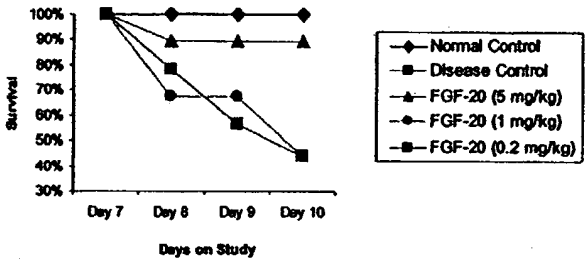

Figure 5. Effect of therapeutically administered FGF-20 on survival in the DSS model of colitis. Female Balb/c mice were exposed to 4% DSS in drinking water for 7 days (day 0 to 6) and then switched to normal drinking water for 4 additional days (day 7 to 10). Disease control animals ($n = 9$) received daily SC injections of vehicle solution on day 4 to day 9. FGF-20 groups ($n = 9$) received daily SC injections of the indicated concentrations of FGF-20 on day 4 to 9. Normal control animals ($n = 3$) were not exposed to DSS, but did receive daily SC injections of vehicle solution on day 4 to 9. Animal survival was recorded daily, and the experiment was concluded on day 10. Note that the disease control and the 0.2 mg/kg FGF-20 groups yielded identical results and are both represented by red squares.

protein products exert a protective effect in intestinal inflammation.[23,24] The result of this experiment indicates that FGF-20 (100 ng/mL) stimulates the expression of both of these genes in HT-29 and Caco-2 human colonic epithelial cancer cells (Figure 10). Peak up-regulation was seen following exposure of cells to FGF-20 for 1–3 hours (COX-2) or 3–6 hours (ITF). An increase in COX-2 protein expression after exposure of HT-29 and Caco-2 cells to FGF-20 for 3 hours was evident via Western blot analysis (data not shown).

Because prostaglandins have been implicated in mucosal healing[23] and PGE_2 production is stimulated by COX-2, we examined the effect of FGF-20 on PGE_2 levels in HT-29 and Caco-2 cells. The result of this experiment indicates that FGF-20 significantly enhances the levels of PGE_2 in a dose-dependent fashion in both of these cell lines. The highest FGF-20 dose examined (100

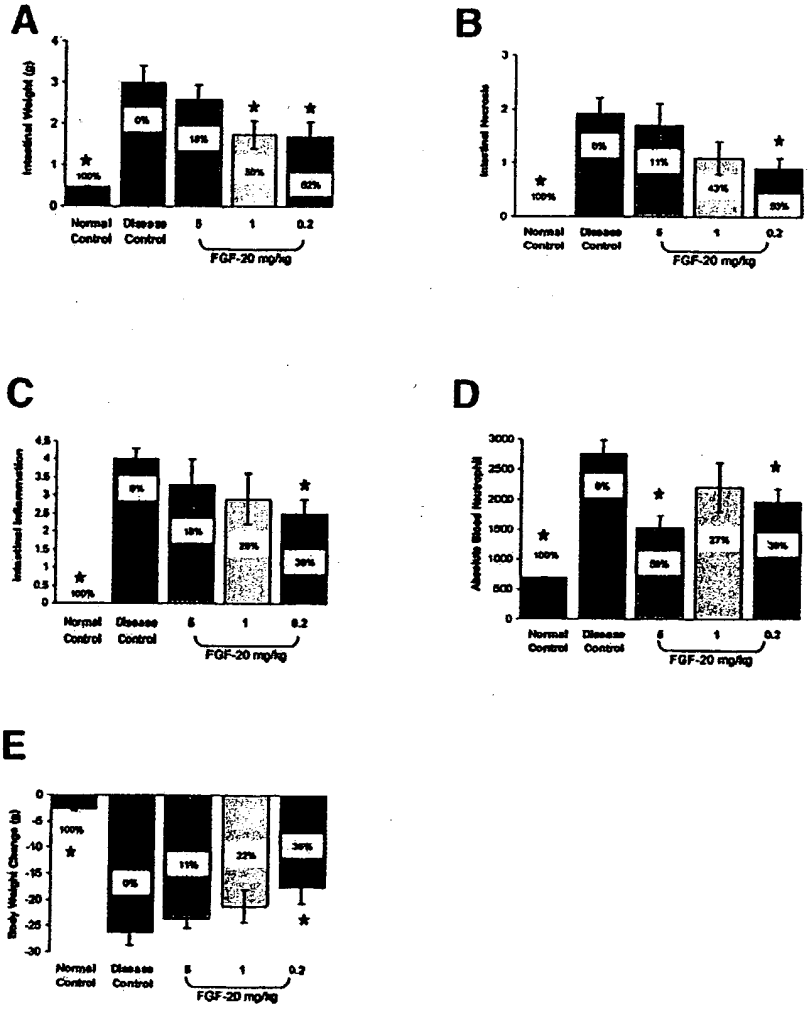

Figure 6. Effects of FGF-20 on indomethacin-induced intestinal ulcerations. To induce intestinal lesions, female Lewis rats were given indomethacin (7.5 mg/kg) SC for 2 days (day 0 to 1). Disease control animals ($n = 8$) received daily IV injections of vehicle solution on day −1 to day 4. FGF-20–treated groups ($n = 8$ animals/group) received daily IV injections of FGF-20 at the indicated concentrations on day −1 to day 4. Normal control animals ($n = 4$) did not receive indomethacin, but did receive daily IV injections of vehicle solution on day −1 to 4. Animals were sacrificed on day 5, and a 10 cm section of the distal jejunum in the area at risk for lesions was (*A*) weighed and histologically examined and scored for level of (*B*) necrosis and (*C*) inflammation. (*D*) The absolute neutrophil counts obtained from blood harvested at necropsy. (*E*) The change in total body weight from day 0 to 5. Results are reported as mean ± standard error, and the percentage changes indicated are relative to the disease control group. Changes that are statistically different from the disease control group ($P < 0.05$ by the 2-tailed Student *t* test) are indicated with a star.

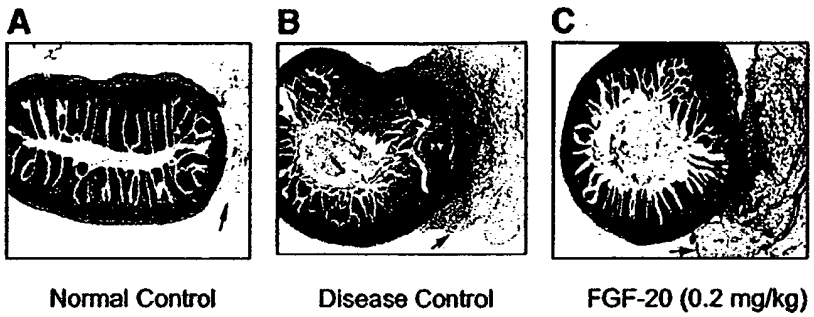

Figure 7. Effects of FGF-20 on indomethacin-induced intestinal ulcerations: histopathology. Representative sections of the distal jejunum from the area at risk were collected at necropsy, preserved in formalin, stained with H&E, magnified 25×, and photographed. The groups are as described in the legend to Figure 6. *Arrows* indicate attached mesentery. Note that FGF-20 (0.2 mg/kg) inhibits the necrosis associated with indomethacin treatment.

ng/mL) stimulated PGE_2 production to a similar degree as that of the positive control (10% FBS).

Discussion

FGF-20 is a novel growth factor that exhibits proliferative activity on fibroblasts and epithelial cells.[19] Because both of these cell types play important roles in

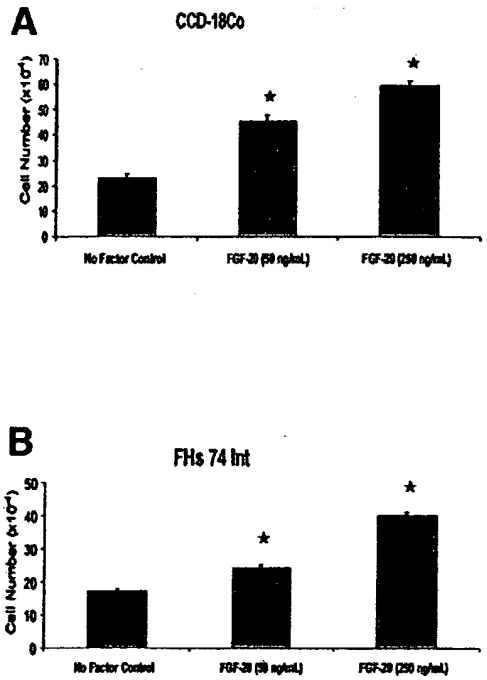

Figure 8. Effects of FGF-20 on the growth of human intestinal cells in vitro. CCD-18Co human colonic fibroblasts (*A*) and FHs 74 Int human intestinal epithelial cells (*B*) were cultured without or with the indicated concentrations of FGF-20 for 6 days and then counted. Data points represent the mean of duplicate wells ± standard deviation. Changes that are statistically different from control cells receiving no factor ($P < 0.05$ by the 2-tailed Student *t* test) are indicated with a star. The experiment was performed 2 times with similar results.

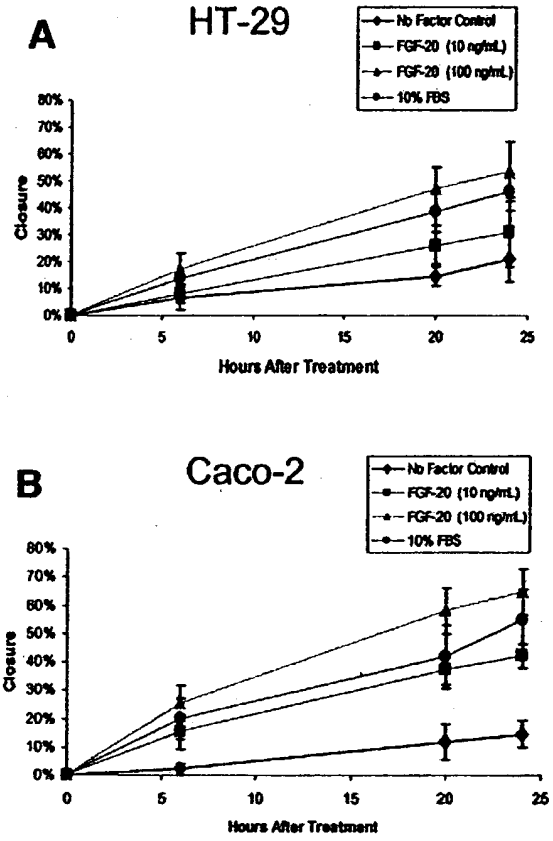

Figure 9. Effects of FGF-20 on the restitution of human colonic cells in vitro. Monolayers of "wounded" HT-29 (*A*) and Caco-2 (*B*) human colonic epithelial cancer cells were cultured without or with the indicated concentrations of FGF-20, or 10% FBS as a positive control, and wound width was measured after 0, 6, 20, and 24 hours. Each data point represents the mean of 12 wounds ± standard deviation, and results are reported as the percentage of closure relative to values obtained at time 0. All FGF-20 data points, with the exception of the treatment of HT-29 with 10 ng/mL FGF-20 for 6 hours, are statistically different ($P < 0.05$ by the 2-tailed Student *t* test) from control cells receiving no factor. The experiment was performed 3 times with similar results.

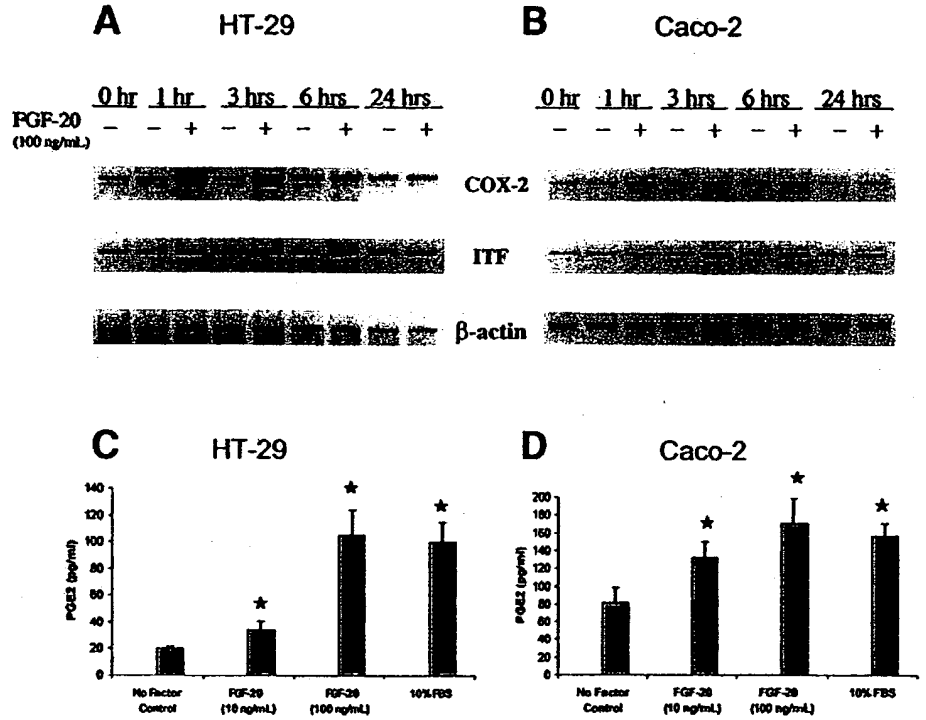

Figure 10. Effects of FGF-20 on the expression of COX-2 and ITF mRNA and on PGE_2 levels in human colonic cells in vitro. The expression of COX-2, ITF, and β-actin RNA transcripts in HT-29 (*A*) and Caco-2 (*B*) human epithelial cancer cells cultured without or with FGF-20 (100 ng/mL) for the indicated time periods was determined by RT-PCR. PGE_2 levels in the media of HT-29 (*C*) and Caco-2 (*D*) cells cultured for 24 hours without or with the indicated concentrations of FGF-20, or 10% FBS as a positive control, were determined by ELISA. Each data point represents the mean of 9 wells ± standard deviation. Changes that are statistically different from control cells receiving no factor ($P < 0.05$ by the 2-tailed Student *t* test) are indicated with a star. The experiment was performed 3 times with similar results.

tissue repair,[4] we sought to examine the effect of FGF-20 in a tissue repair model. To this end, we initiated a series of experiments to explore the effect of FGF-20 in animal models of IBD, a disease in which the integrity of the intestinal epithelium is compromised[1–3] and for which additional therapies are needed. Further rationale for assessing the activity of FGF-20 in IBD animal models originates from the finding that FGF-20 interacts strongly with various FGFRs, including FGFR2b, FGFR2c, and FGFR3c,[19] at least 1 of which is present on intestinal epithelium,[7,8] and from previous studies implicating FGFs in intestinal epithelial repair.[7,13–18,25]

The present findings demonstrate that FGF-20 is active in 2 independent rodent IBD models: DSS treatment of mice to induce an ulcerative colitis-like syndrome, and indomethacin treatment of rats to induce a Crohn's-like disease consisting of inflammation and ulceration of the small bowel. The decision to use these 2 models was based on the fact that each model affects a different region of the gastrointestinal tract and thus may represent different human disease counterparts. Moreover, the pathologies associated with these models are highly reproducible.[22]

In the DSS model, a significant beneficial effect of FGF-20, as indicated by various experimental parameters, was evident when this growth factor was administered concomitantly with DSS. This effect was seen regardless of whether FGF-20 was administered through the IP or the SC route. In addition, a beneficial effect of FGF-20 on animal survival was found when FGF-20 was administered therapeutically after 4 days of DSS exposure. The protective effects of FGF-20 in the DSS model occurred in a dose-dependent fashion, with maximum protection observed at the highest concentration examined (5 mg/kg). FGF-20–administered IV also proved active in the rat indomethacin model, in which an inverse dose-response was observed. Bimodal dose-response curves have been reported for other biological molecules,[13,26,27] and it is possible that the activity of FGF-20 in the DSS and indomethacin models fall on opposite sides of the response curve.

The in vitro studies presented herein suggest multiple mechanisms for explaining how FGF-20 stimulates intestinal healing. For example, FGF-20 may be enhancing mucosal repair, a hypothesis supported by

the finding that this factor increases the restitution of colonic epithelial cells and the growth of colonic fibroblasts and intestinal epithelial cells. Because ITF has been shown to accelerate epithelial restitution[24,28] and FGF-20 increases ITF expression in colonic epithelia, it is also possible that ITF mediates at least some of the mucosal repair induced by FGF-20. Additional properties of ITF that may contribute to the support of the mucosal barrier include its ability to increase mucus viscosity[29] and prevent epithelial cell apoptosis.[30] The data further suggest that FGF-20 may enhance mucosal healing by stimulating a COX-2–mediated increase in PGE_2, a molecule which has been shown to stimulate the healing process.[23,31] The net effect of accelerated epithelial restitution, restoration of an intact epithelial layer, and improved mucosal barrier function after FGF-20 treatment is decreased mucosal permeability and reduced inflammation caused by decreased uptake of inflammation-inducing substances, including bacterial antigens, cell wall polymers, and chemotactic peptides.[1] Moreover, PGE_2 (generated by COX-2 by way of FGF-20) may reduce inflammation by inhibiting inflammatory cell activation.[32]

There are currently no marketed drugs for IBD that stimulate intestinal repair, although at least 2 FGF family members (FGF-7 and FGF-10, also known as KGF-1 and KGF-2, respectively), have shown activity in animal models of IBD.[13,17] Because of their narrow receptor specificity, FGF-7 and FGF-10 efficiently activate some epithelial cells, but not fibroblasts.[33] However, FGF-20 interacts with a variety of FGF receptors[19] and thus is capable of activating fibroblasts as well as epithelial cells. Evidence suggests that fibroblasts play an important role in the repair of epithelium,[4] and thus FGF-20 may be well suited for the treatment of this disease.

A biological agent for the treatment of IBD, an antibody to TNF-α, has proven useful in the treatment of moderate to severe Crohn's disease.[34] However, use of this product increases the risk of infection.[34] In contrast, FGF-20 represents a new class of biological agent that may prove effective in the treatment of IBD by stimulating intestinal repair without increasing the risk of infection.

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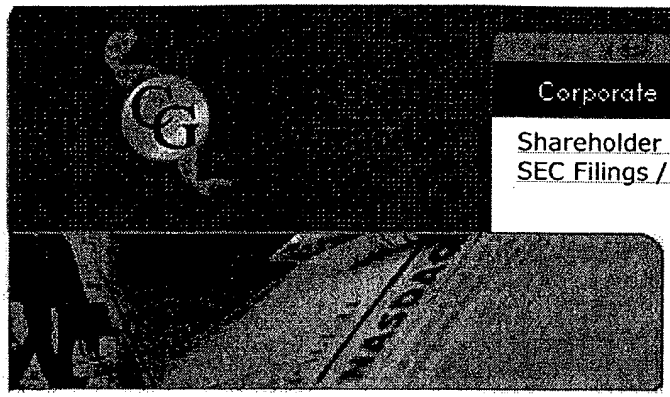

Press Releases

CuraGen Corporation (ticker: CRGN, exchange: NASDAQ) News Release - 4-Mar-2003

CuraGen Receives FDA Approval to Initiate Clinical Trials

Potential Oral Mucositis Treatment Marks Successful Transition into Drug Development

NEW HAVEN, Conn., Mar 4, 2003 /PRNewswire-FirstCall via COMTEX/ --CuraGen Corporation (Nasdaq: CRGN), a genomics-based pharmaceutical company, today announced that the U.S. Food and Drug Administration (FDA) has approved its Investigational New Drug (IND) application to initiate clinical trials for CG53135, a potential protein therapeutic being investigated as a treatment for oral mucositis. Oral mucositis is a side effect of chemotherapy and radiotherapy that results in the degradation of mucosal tissue that can range from redness and irritation to severe ulcerations of the mouth and throat. CuraGen now plans to proceed with a multi-center Phase I clinical trial to evaluate safety and pharmacokinetics in patients with cancer who are at risk for mucositis following chemotherapy.

Mucositis is a debilitating complication of cancer chemotherapy or radiotherapy that affects the mucosal tissue, which acts as a protective lining within the digestive track, including the mouth and throat. Symptoms range from pain and discomfort to severe ulcerations that limit a patient's ability to ingest nutrients. Mucositis can result in a suppressed immune system that can reduce a patient's ability to tolerate further cancer therapy. Delayed treatment can lessen the effectiveness of the chemotherapy or radiotherapy, adversely impacting the value of the patient's overall treatment regimen.

"CG53135 is a novel protein discovered through the application of CuraGen's functional genomic technologies. In preclinical studies, this potential protein therapeutic reduced tissue inflammation and degeneration, and minimized the severity and extent of mucosal tissue damage. Mucositis is a significant unmet medical need, and we are pleased to have the opportunity to advance this promising molecule into human clinical trials," stated Timothy M. Shannon, M.D., Senior Vice President of R&D and Chief Medical Officer of CuraGen Corporation.

"Through the filing of this IND, CuraGen has become one of the first genomics companies to successfully transition from a target discovery company into a genomics-based pharmaceutical company. This molecule represents the first of many promising candidates that we believe will emerge from our portfolio of discovery and preclinical stage projects. We are pleased with the progress of this potential therapeutic and look forward to additional future successes," stated Jonathan M. Rothberg, Ph.D., Founder, Chairman, and CEO of CuraGen Corporation.

About CuraGen

CuraGen Corporation (NASDAQ: CRGN) is a genomics-based pharmaceutical company.

CuraGen's integrated, functional genomic technologies and Internet-based bioinformatic systems are designed to generate comprehensive information about genes, human genetic variations, gene expression, protein interactions, protein pathways, and potential drugs that affect these pathways. The Company is applying its industrialized genomic technologies, informatics, and validation technologies to develop protein, antibody, and small molecule therapeutics to treat obesity and diabetes, cancer, inflammatory diseases, and central nervous system (CNS) disorders. CuraGen is headquartered in New Haven, CT and additional information is available at www.curagen.com.

This press release may contain forward-looking statements including statements about CG53135's demonstrated ability to reduce tissue inflammation and degeneration, and minimize the severity and extent of mucosal tissue damage in preclinical studies, as well as representing the first of many promising candidates that we believe will emerge from our portfolio of discovery and preclinical stage projects. Such statements are based on management's current expectations and are subject to a number of factors and uncertainties that could cause actual results to differ materially from those described in the forward-looking statements. CuraGen cautions investors that there can be no assurance that actual results or business conditions will not differ materially from those projected or suggested in such forward-looking statements as a result of various factors, including, but not limited to, the following: CuraGen's expectation that it will incur operating losses in the near future, the early stage of development of CuraGen's products and technologies, uncertainties related to preclinical and clinical testing and trials, uncertainties and adverse results relating to CuraGen's ability to obtain regulatory approval for its products in development, uncertainties surrounding the availability of additional funding, CuraGen's reliance on research collaborations and strategic alliances, the actions of competitors, the development of competing technologies, CuraGen's ability to protect its patents and proprietary rights, patent infringement actions and uncertainties relating to commercialization rights. Please refer to our Annual Report on Form 10-K for the fiscal year ended December 31, 2001 for a description of these risks. We disclaim any intention or obligation to update or revise any forward-looking statements, whether as a result of new information, future events, or otherwise.

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Exam Initials	Cite No.	U.S. Patent Document No.	Issue Date	Name of Patentee(s) or Applicant(s)	Class	Sub Class	Filing Date If Appropriate
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	B8	WO	01/92522	Eli Lilly and Co.	December 6, 2001	X	
	B9	WO	02/24234	The Regents of the University of California	March 28, 2002	X	

OTHER PRIOR ART – NON PATENT LITERATURE DOCUMENTS

Exam Initials	Cite No.	Name of Author, Title (when appropriate), Publication, Volume, Page(s), Date, Etc.
	C21	Bange et al. (2002). *Cancer Res* 62: 840-847.
	C22	Ohmachi et al. (2000). *Biochemical and Biophysical Res Comm* 277: 355-360.
	C23	Wong et al. (2001). *Am J of Medical Genetics* 102: 282-285.
	C24	International Search Report for PCT/US02/19400, mailed June 4, 2003

* a copy of this reference is not provided as it was previously cited by or submitted to the office in a prior application, Serial No. _____, filed _____, and relied upon for an earlier filing date under 35 U.S.C. §120 (continuation, continuation-in-part, and divisional applications).

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